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Mouse-Protecting and Histamine-Sensitizing Activities of Pertussigen and Fimbrial Hemagglutinin from *Bordetella pertussis*

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We compared the protective activities of fimbrial hemagglutinin (FHA) and pertussigen and their respective antibodies in mice infected intracerebrally with *Bordetella pertussis*. We found that mice were protected by a 1.7-μg/mouse dose of pertussigen which was free of detectable FHA and was detoxified by treatment with glutaraldehyde. A pertussigen preparation made from cells grown in shake cultures that did not contain demonstrable FHA protected mice at a dose of 1.4 μg/mouse. FHA at a dose of 10 μg/mouse protected mice from intracerebral infection, but it also sensitized mice to histamine at a dose of 2 μg/mouse, which indicated that it was contaminated with pertussigen. When FHA was obtained free of demonstrable pertussigen, it failed to sensitize mice to histamine at a dose of 30 μg/mouse and to protect mice from infection at a dose of 12 μg/mouse (largest doses tested). Passive protection tests with antisera known to contain antibodies to pertussigen protected mice from intracerebral infection, whereas sera lacking anti-pertussigen antibodies but containing high concentrations of anti-FHA antibodies did not protect mice. The most important antigen for the immunization of mice against intracerebral infection with *B. pertussis* appears to be pertussigen.

Keogh et al. (8, 9) demonstrated that *Bordetella pertussis* produces a substance capable of agglutinating erythrocytes from various animals, including birds and reptiles. These workers showed that preparations containing hemagglutinin (HA) induced active immunity in mice against intranasal infection with *B. pertussis*. Soon after these studies, Masry (12) demonstrated that HA was not the mouse-protective antigen when mice were challenged by the intracerebral (i.c.) route. These observations were confirmed by Thiele (33) and Pillemer (24). The British Medical Research Council studies on whooping cough vaccines established a good correlation between the mouse-protective activity of a vaccine, as measured by i.c. challenge, and its effectiveness in immunizing children (13, 14). For this reason in the United States and many other countries a protection test using i.c. challenge has been adopted as the standard method for measuring the protective potencies of vaccines (4, 34).

The work of Masry (12), Thiele (33), and Pillemer (24) and subsequent work done in our laboratory (18) seemed to rule out HA as a mouse-protective antigen when the challenge was given i.c. Recently, Sato et al. (31), Arai and Sato (3), and Irons and MacLennan (7) reported that *B. pertussis* produces two HAs. Arai and Sato (3) called these fimbrial HA (FHA) and leukocytosis-promoting factor HA. These substances were separated from leukocytosis-promoting factor preparations previously thought to be pure (28-30). Arai and Sato (3) and Sato et al. (31) showed that FHA was nontoxic and protected mice from i.c. challenge with *B. pertussis*, whereas leukocytosis-promoting factor HA was toxic and nonprotective at the doses tolerated by mice. Irons and MacLennan (7) confirmed these observations. In our laboratory however, we have observed repeatedly that fractions lacking HA can protect mice (16, 18). These preparations, which were made with acetone-extracted cells (18) or with cells extracted with buffer containing Triton X-100 (16), were tolerated by mice at doses of at least 10 μg/mouse, whereas leukocytosis-promoting factor HA made by the method of Arai and Sato (3) or as described below was toxic at doses of 0.1 to 0.5 μg/mouse. For reasons given previously (16), we call leukocytosis-promoting factor HA pertussigen, and in our view pertussigen is similar to leukocytosis-promoting factor HA, to the histamine-sensitizing factor (16), to the islet-activating protein (35), to the pertussis toxin (25), and to the late-appearing toxic factor of *B. pertussis*

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(10). In an effort to clarify the roles of FHA and pertussigen in the protection of mice from i.c. infections, we investigated the effectiveness of these two substances and their corresponding antibodies in the protection of mice from experimental i.c. infections with virulent *B. pertussis*.

MATERIALS AND METHODS

Preparation of FHA free of detectable pertussigen. FHA was prepared by a previously described method (1). The resulting preparations still contained pertussigen, since they sensitized mice to histamine at doses of 2 to 10 μg/mouse and induced leukocytosis at doses of 20 to 50 μg/mouse, depending on the preparation. To prepare FHA free of pertussigen, the following procedure was followed.

To 1.5 mg of FHA dissolved in 2.5 ml of 50 mM tris(hydroxymethyl)aminomethane (Tris)–1 M NaCl (pH 8), 20 μl of 10% Triton X-100 was added (final concentration, 0.1%). This mixture was allowed to stand at room temperature for 2 h and then chromatographed in a Sephacryl S-200 column (41 by 0.67 cm) in 50 mM sodium acetate buffer (pH 6.8) containing 0.5 M NaCl and 0.01% Triton X-100; 30-drop fractions were collected, the optical densities at 280 nm were determined, and the hemagglutinating activities were assayed. The fractions containing the greatest amounts of FHA, as measured by agglutination of chicken erythrocytes, were pooled. This pool was fully active by the hemagglutination test, but had no histamine-sensitizing activity at 29 μg of extract per mouse, which was the highest dose tested.

Preparation of pertussigen free of detectable FHA. *B. pertussis* 3779BL_2S_4 (agglutinogen types 1, 3, and 6) was grown for 48 h at 37°C with constant shaking in the Casamino Acids liquid medium described previously (16). Under these conditions, undetectable amounts of FHA were produced, as judged by hemagglutination tests (2, 18). The cells were collected and extracted with 1 M NaCl–0.05 M sodium pyrophosphate buffer (pH 8) containing 0.5% Triton X-100 as described previously (19). The extract was dialyzed extensively against distilled water (daily 20-liter changes for 7 days) until a floccular precipitate appeared. (The pH of the distilled water was lowered to 4.5 with HCl during the last 2 days of dialysis.) The precipitate was collected by centrifugation at 16,000 × *g* for 0.5 h. The precipitate was extracted with 0.5 M NaCl–0.01 M sodium pyrophosphate–0.017% Triton X-100 (pH 7.4). The extract was designated TEP-III and was chromatographed through a Bio-Gel A.5 column (2.6 by 40 cm) equilibrated in the same buffer. Most of the activity, as measured by induction of histamine hypersensitivity in mice, was found in the second protein peak. The pool (731 ml) of active fractions was concentrated by ultrafiltration under vacuum to 30 ml (0.520 mg of protein per ml) and designated pool B. The histamine-sensitizing dose (50% sensitizing dose) of pool B was about 15 ng of protein per mouse. As determined by gel diffusion and hemagglutination, this material was free of FHA.

Pertussigen was also obtained in a crystalline form, either by a method described elsewhere (2a) or by the following method. *B. pertussis* 3779BL_2S_4 was cultured under stationary conditions for 5 days at 35°C in the synthetic liquid medium of Stainer and Scholte (32) containing 12 mM Tris base. Usually, 20-liter lots were used (500 ml per 4-liter Erlenmeyer flask). Thimerosal was added at the end of the incubation period to a final concentration of 1:10,000. The cultures were then centrifuged at 1,600 × *g* for 1 h. The supernatant fluids were collected, and the sediments were discarded. Pertussigen was precipitated from the clear supernatant fluid by a modification of the method described by Niwa (23); this involved lowering the pH to 6.5 with glacial acetic acid and adding 20 ml of a $ZnCl_2$ solution (0.5 g/ml) per liter of supernatant fluid. This mixture was stirred for 2 h at 2 to 5°C and left overnight at the same temperature. The precipitate was collected by centrifugation at 1,600 × *g* for 0.5 h. The inactive supernatant was discarded. The Zn-protein complex was dissociated by adding 37 ml of a 20% solution of Na_2HPO_4 per liter of original culture supernatant. The mixture was stirred for 0.5 h at room temperature and centrifuged at 1,600 × *g* for 0.5 h. The supernatant was saved, and the sediment was extracted twice with 37 ml of 50 mM Tris–1 M NaCl (pH 7.6) (TNB). After each extraction the mixture was stirred for 15 min at room temperature and then centrifuged as described above. The extracts were pooled with the Na_2HPO_4 supernatant and incubated at 2 to 5°C overnight. Much of the Na_2HPO_4 crystallized out of solution during this incubation and was removed. This extract was kept frozen until needed.

A 650-ml portion of this extract was dialyzed at 2 to 5°C against three successive changes of 16 liters of 5 mM Tris HCl (pH 8). The precipitate that developed was collected by centrifugation at 1,600 × *g* for 1 h. The supernatant was discarded. To the precipitate, 20 ml of 0.1 M sodium pyrophosphate (pH 10.5) was added; this mixture was stirred, and then 20 ml of TNB was added to lower the pH to 8.4 This mixture was centrifuged at 1,600 × *g* for 0.5 h. The supernatant was saved, and the sediment was extracted twice more by adding 10 ml of 0.1 M sodium pyrophosphate and then 10 ml of TNB; the mixture was centrifuged as described above, and the supernatant was pooled with the first extraction. The extract was passed through a Sepharose CL-6B column (2.5 by 90 cm) equilibrated with TNB containing 5% ethanol. The active fractions, as measured by agglutination of chicken erythrocytes, were pooled (total volume, 130 ml) and concentrated by ultrafiltration under vacuum to 4.7 ml. This material was then passed through a Bio-Gel A.5 column (1.5 by 90 cm) also equilibrated with TNB containing 5% ethanol. The active fractions were pooled (total volume, 18 ml) and concentrated by ultrafiltration to 3.2 ml. The optical density at 280 nm of this concentrated solution was 1.15, which corresponded to approximately 0.57 mg of protein per ml. When this solution was kept at 2 to 5°C for 2 to 3 weeks, crystals of pertussigen developed.

Detoxification of pertussigen. A solution of pertussigen purified as described above was made in 20 mM sodium phosphate containing 0.5 M NaCl (pH 7.6). This solution had an optical density at 280 nm of 0.228, which was equivalent to 114 μg of protein per ml. Enough 0.2% glutaraldehyde solution (made in the same buffer) was added to bring the final concentra-

tion of glutaraldehyde to 0.05%. The mixture was incubated at room temperature for 2 h, and then enough 0.2 M L-lysine solution (made in same buffer) was added to bring the final concentration of L-lysine to 0.02 M. The mixture was incubated for 2 h at room temperature and then dialyzed for 2 days against 20 mM sodium phosphate buffer containing 0.5 M sodium chloride and 0.02 M L-lysine.

Assay methods. Hemagglutination tests, mouse protection tests, and tests for induction of histamine hypersensitivity were performed as previously described (16). The passive protection test was performed as described by Sato et al. (30).

Protein determinations were done either by measuring optical density at 280 nm or by the method of Lowry et al. (11), using the Folin-Ciocalteau reagent.

Gel diffusion tests were performed in 1% agarose dissolved in 50 mM Tris–1 M NaCl (pH 8).

Animals. The mice used were the outbred CFW strain and were raised in our animal production unit. For active protection tests 3-week-old male mice were used, for passive protection tests 5-week-old male mice were used, and for histamine sensitization tests 6- to 8-week-old female mice were used. Albino rabbits and sheep were purchased from a local supplier.

Preparation of antisera. Rabbits were each immunized with 100 μg of purified FHA emulsified in complete Freund adjuvant (0.5 ml of FHA plus 0.5 ml of adjuvant), which was distributed into the two hind footpads, and 21 days later they received intravenously 100 μg of FHA dissolved in TNB. These animals were bled 7 days later. Rabbits were also immunized with the pool B pertussigen preparation, which was known to be free of demonstrable FHA by gel immunodiffusion tests; 2 ml of pool B (optical density at 280 nm, 2.16) was adsorbed onto 2 ml of washed and packed rabbit erythrocytes. The cells were washed again to remove nonabsorbing materials, and the washed cells containing pertussigen were suspended in 4 ml of saline. Each rabbit received 1 ml of this suspension by the footpad route (0.5 ml/footpad). The exact amount of active material given to each rabbit was not known. The rabbits were each boosted intravenously with a similar dose of material at 4, 5, 6, and 7 weeks after the original injection, and they were bled 2 weeks after the last booster dose. A sheep was immunized subcutaneously with 168 μg of highly purified pertussigen prepared as described previously (1) and adsorbed to 2.8 mg of sheep erythrocyte stromata (weight is expressed as protein). Similar doses were given intravenously 4, 6, and 9 weeks later, and a subcutaneous dose was given 12 weeks later. The sheep was bled 2 weeks after the last booster dose.

RESULTS

Protective activities of FHA and pertussigen preparations. The following four different preparations were tested for the ability to immunize mice against i.c. challenges with *B. pertussis*: (i) a crystalline preparation of FHA known to contain pertussigen activity; (ii) a preparation of FHA free of detectable pertussigen activity; (iii) pertussigen (pool B) prepared from cells grown in shake cultures and known to be free of detectable FHA activity; and (iv) a detoxified pertussigen preparation without detectable FHA activity. The protection test (Table 1) showed that the FHA contaminated with pertussigen protected mice at a dose of 10 μg/mouse (50% protective dose), whereas the FHA preparation free of pertussigen failed to protect at a dose of 12 μg/mouse (highest dose tested). On the other hand, the two preparations of pertussigen known to be free of FHA protected mice at doses of 1.4 μg/mouse (pool B) and 1.7 μg/mouse (detoxified pertussigen). Table 2 shows the hemagglutinating and histamine-sensitizing activities of these preparations, as well as the activities of a preparation of FHA free of pertussigen supplied by J. Cowell (Bureau of Biologics, Bethesda, Md.) and a fully toxic preparation of crystalline pertussigen. A 2-μg/mouse dose of crystalline FHA sensitized mice to histamine, whereas pertussigen sensitized mice at a dose of 0.0027 μg/mouse. The two preparations of FHA known to be free of pertussigen and the detoxified pertussigen preparation did not sensitize mice to histamine at the highest doses tested (30, 20, and 0.016 μg/mouse, respectively). It should be emphasized that contamination of crystalline FHA with 0.13% pertussigen could have accounted for the histamine-sensitizing activity of FHA. The two preparations of FHA made by us had high hemagglutinating activities, whereas the pertussigen preparation had a lower activity, which was of the granular type (Table 2). The detoxified pertussigen preparation did not agglutinate erythrocytes at the highest concentration tested.

Passive protection tests with anti-FHA and anti-pertussigen sera. The following five different sera were tested for the ability to protect mice from i.c. infections with *B. pertussis*: (i) an antiserum which was obtained from a sheep immunized with a preparation of pertussigen slightly contaminated with FHA and contained both anti-pertussigen and anti-FHA antibodies; (ii) an antiserum which was prepared in rabbits against pertussigen and contained no demonstrable anti-FHA antibodies; (iii) a rabbit anti-FHA serum which contained no demonstrable anti-pertussigen antibodies; (iv) a normal sheep serum; and (v) a normal rabbit serum (Fig. 1 and 2). Each mouse received 0.5 ml of a serum dilution intraperitoneally 2 h before an i.c. challenge with approximately 30,000 viable *B. pertussis* cells (Table 3). The sheep antiserum containing both anti-FHA and anti-pertussigen antibodies protected 50% of the mice at a dilution of 1:8.1 (50% protective dose). The rabbit anti-pertussigen serum containing no anti-FHA

TABLE 1. *Active protection tests with FHA and pertussigen*[a]

Prepn	Dose (μg of protein per mouse)	No. of survivors/no. tested[b]	PD_{50} (μg/mouse)[c]	Hemagglutinating activity (HAU/mg)[d]
Crystalline FHA	0.00	1/16	10	1.5×10^5
	2.00	2/16		
	10.00	9/16		
	50.00	12/16		
FHA free of detectable pertussigen	0.00	3/16	≫12	0.9×10^5
	0.48	3/16		
	2.40	5/16		
	12.00	1/16		
Pertussigen (pool B)[e]	0.00	1/16	1.4	ND[f]
	0.40	5/16		
	2.00	9/16		
	10.00	13/16		
Pertussigen detoxified with glutaraldehyde	0.00	0/10	1.7	ND
	0.08	0/10		
	0.40	2/10		
	2.00	5/10		
	10.00	10/10		

[a] Each mouse received intraperitoneally the dose indicated and 14 days later was challenged i.c. with approximately 30,000 *B. pertussis* cells.

[b] Number of survivors/total number tested 14 days after challenge.

[c] PD_{50}, Dose that protected 50% of mice challenged.

[d] One hemagglutinating unit (HAU) was the minimum amount of substance that agglutinated the erythrocytes in 50 μl of a 0.5% suspension.

[e] Prepared from cells extracted with 0.5% Triton X-100–1 M sodium chloride–0.5 M sodium pyrophosphate (pH 8). This preparation was still impure but contained no detectable FHA and was well tolerated at the highest dose tested.

[f] ND, Not detected.

antibodies protected 50% of the mice at a dilution of 1:1.9, whereas the rabbit anti-FHA serum, which was by far the most potent serum (as judged by gel radial diffusion tests [Fig. 3]), did not show protection when given undiluted. Normal sheep and rabbit sera did not give any protection.

HAs from *Bordetella bronchiseptica* and *Bordetella parapertussis*. Ross et al. (27) showed that *B. pertussis* vaccines protected mice from infection with *B. bronchiseptica* but that vaccines made from *B. bronchiseptica* did not protect against *B. pertussis*. They also showed that the antigen responsible for protection in the *B. pertussis* vaccine was heat labile, whereas the antigens in *B. bronchiseptica* and *B. parapertussis* responsible for inducing protection against *B. bronchiseptica* were heat stable. It is known that these three species of *Bordetella* have agglutinogens in common (6). In addition, they produce FHA (9). In this work we demonstrated that *B. parapertussis* grown under the conditions and in the media described by Sato et al. (30) for *B. pertussis* produced agglutinin for chick erythrocytes in titers lower than those produced by *B. pertussis* cultures. After these supernatants were concentrated by ultrafiltration under vacuum, we found that this HA was serologically identical to the FHA of *B. pertussis* (Fig. 4). These results and those of protection tests performed with whole-cell vaccines provide additional evidence that FHA may not be protective for mice when these animals are challenged i.c. with *B. pertussis*.

DISCUSSION

Ever since the development of an effective pertussis vaccine, workers have attempted to purify the protective antigen from *B. pertussis* cells. Little progress has been made, perhaps due to peculiarities of the antigen, but mainly due to unsatisfactory assay methods. It is still doubtful that the mouse protection test used to measure the presence of this substance is a reliable method for measuring the antigen that induces immunity against whooping cough in children (26). With whole-cell vaccines a good correlation has been found between mouse protection and protection of children (13, 14), but with pure antigens this may not necessarily be the case.

For many years we have observed that only fractions of *B. pertussis* cells that contain pertussigen activity (histamine-sensitizing factor, leukocytosis-promoting factor) protect mice from i.c. infection with *B. pertussis* (16). We and

TABLE 2. *Histamine-sensitizing activities of FHA and pertussigen*

Prepn	Dose (μg of protein per mouse)	No. of deaths/total no.[a]	SD_{50} (μg/mouse)[b]	Hemagglutinating activity (HAU/mg of protein)[c]
Crystalline FHA	0.00	0/7	2	1.5×10^5
	0.40	1/7		
	2.00	3/7		
	10.00	7/7		
FHA free of detectable pertussigen	0.00	1/5	>30	2.56×10^5
	0.24	0/5		
	1.20	1/5		
	6.00	0/5		
	30.00	1/5		
FHA free of detectable pertussigen[d]	0.00	1/10	>20	8×10^3
	0.80	2/10		
	4.00	0/10		
	20.00	0/10		
Pertussigen (pool B)[e]	0.00	2/10	0.064	ND[f]
	0.056	5/10		
	0.280	9/10		
	1.400	10/10		
Crystalline pertussigen	0.00	0/10	0.0027	5.3×10^{3g}
	0.00064	0/10		
	0.0032	6/10		
	0.0160	9/10		
Pertussigen detoxified with glutaraldehyde	0.00	0/10	>0.016	ND
	0.00064	1/10		
	0.0032	2/10		
	0.0160	1/10		

[a] Deaths due to 0.5 mg of histamine/total number of mice challenged.

[b] SD_{50}, Dose that induced sensitization of 50% of the mice challenged intraperitoneally with 0.5 mg of histamine given 3 days later.

[c] The hemagglutination due to FHA was characterized by formation of a smooth film on the bottom of the wells. HAU, Hemagglutinating units.

[d] This sample was supplied by J. Cowell, Bureau of Biologics, Bethesda, Md. It was received in 2.5 mM Tris-hydrochloride (pH 8) containing 0.5 M NaCl and 50% glycerol. It was kept at −15°C for a few weeks. The original HA titer was not known.

[e] Prepared from cells extracted with 0.5% Triton X-100–1 M sodium chloride–0.5 M sodium pyrophosphate (pH 8).

[f] ND, Not detected.

[g] The hemagglutination due to pertussigen was characteristically granular in appearance, and the cells did not disperse when shaken.

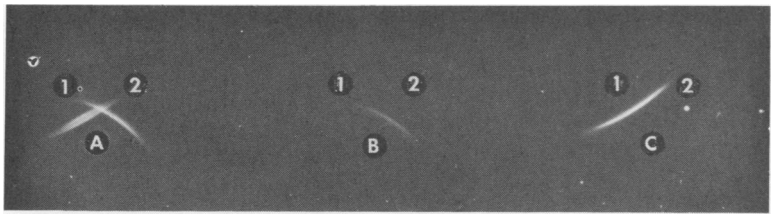

FIG. 1. *Agarose gel immunodiffusion tests. (A) Sheep antiserum containing both anti-pertussigen antibody and anti-FHA antibody, tested against an FHA solution (about 1.1 mg/ml) (well 1) and a pertussigen solution (about 52 μg/ml) (well 2). (B) Rabbit anti-pertussigen serum free of anti-FHA antibody, tested against the same solutions of FHA (well 1) and pertussigen (well 2). (C) Rabbit anti-FHA serum free of anti-pertussigen antibody, tested against the same solutions of FHA (well 1) and pertussigen (well 2).*

others excluded the role of agglutinogen factors, endotoxin, heat-labile toxin, and HA (16) in the protection of mice against i.c. challenge with *B. pertussis*.

Some workers do not think that pertussigen (histamine-sensitizing factor, leukocytosis-promoting factor) and the mouse-protective antigen are identical (5, 21), but the evidence for this has

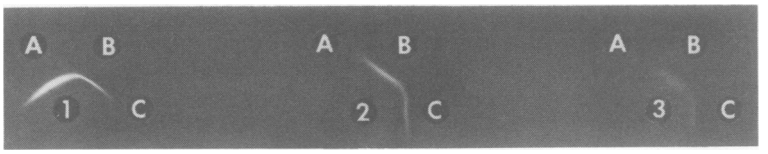

FIG. 2. *Agarose gel immunodiffusion tests. Antigen-containing wells: well 1, FHA (solution containing about 300 μg/ml); well 2, pertussigen (solution containing about 52 μg/ml); well 3, pool B (solution containing 1.3 mg/ml). Antibody-containing wells: well A, rabbit anti-FHA serum; well B, sheep antiserum containing both anti-FHA antibody and anti-pertussigen antibody; well C, rabbit anti-pertussigen serum.*

TABLE 3. *Passive protection tests with anti-FHA and anti-pertussigen sera*[a]

Serum	No. of survivors/no. challenged at the following dilutions			PD_{50}[b]
	1:1	1:5	1:25	
Sheep anti-pertussigen containing anti-FHA antibodies	14/15[c]	10/15	2/15	1:8.1
Rabbit anti-FHA	0/15	1/15	0/15	
Rabbit anti-pertussigen	10/15	2/15	0/15	1:1.9
Normal sheep	2/15	0/15		
Normal rabbit		0/15		

[a] Each mouse received intraperitoneally 0.5 ml of serum dilution indicated 2 h before i.c. challenge with approximately 30,000 viable *B. pertussis* cells. The animals were observed for 14 days.

[b] PD_{50}, Dilution of serum that protected 50% of the mice challenged.

[c] Mice surviving the challenge/total number of mice challenged.

been indirect and inconclusive.

Recently, workers in three laboratories reported that preparations of pure FHA were capable of inducing immunity in mice to i.c. infection with *B. pertussis* (7, 15, 31). The results of Sato et al. (31), and Irons and MacLennan (7), and Morse (15) contradict our previously published data (16, 18, 20) showing that only preparations containing pertussigen are protective for mice when these animals are immunized with a single intraperitoneal dose and challenged i.c. 14 days later.

Recently, we obtained a crystalline preparation of FHA (1) that protected mice (unpublished data), but it was contaminated significantly with pertussigen. Similarly, we found that preparations made by the method of Arai and Sato (3) were also contaminated with pertussigen (unpublished data). The work reported here clearly showed that crystalline preparations of FHA protected mice at a dose of 10 μg/mouse, whereas pertussigen (pool B) free of detectable FHA protected mice at a lower dose (1.4 μg/mouse). It should be noted that pool B was prepared from *B. pertussis* cells extracted with buffer containing Triton X-100, and this preparation was well tolerated by mice at a dose of 10 μg/mouse. Furthermore, when crystalline pertussigen preparations that were too toxic to be tested at doses of more than 0.1 μg/mouse were detoxified with glutaraldehyde, they protected mice at a low dose (1.7 μg/mouse). These preparations contained no demonstrable FHA, as determined by agar gel diffusion. It should be stated that if the detoxified material had not lost any effectiveness, these results would indicate that the FHA preparation had to be contaminated with about 17% pertussigen, which seems to be a high degree of contamination for a crystalline FHA preparation. It is possible that the protective activity of detoxified pertussigen is

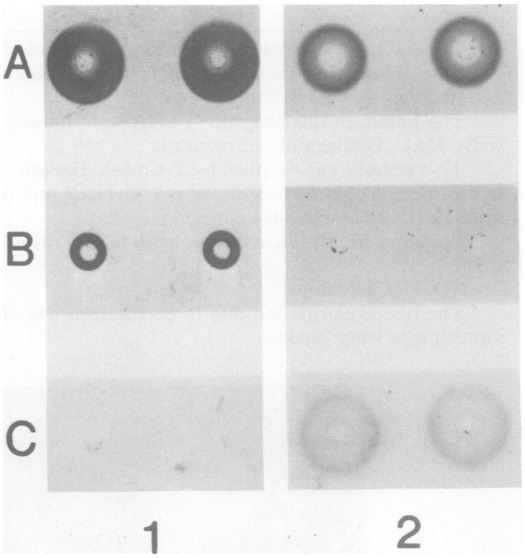

FIG. 3. *Mancini-type radial immunodiffusion in 1% agarose gels made with 0.5 mM Tris (pH 8)–1 M sodium chloride and stained with Coomassie blue. All of the antisera were at final dilutions of 1:50 in the gels. (A) Sheep antiserum containing both anti-FHA and anti-pertussigen antibodies. (B) Rabbit anti-FHA serum. (C) Rabbit anti-pertussigen serum. The duplicate wells in column 1 each contained 3 μl of an FHA solution (concentration, about 300 μg/ml). The duplicate wells in column 2 each contained 3 μl of a pertussigen solution (concentration, about 52 μg/ml).*

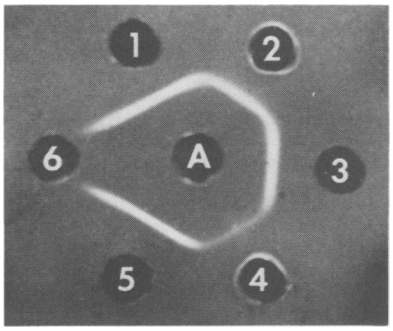

FIG. 4. *Gel diffusion test in 1% agarose made with 50 mM Tris (pH 8)–1 M sodium chloride. The center well (well A) contained rabbit anti-FHA serum. Well 1, FHA prepared from B. pertussis* 3779BL_2S_4; *well 2, FHA prepared from B. parapertussis* 17903; *wells 3 and 5, FHA prepared from B. pertussis Tohama; well 4, FHA prepared from B. bronchiseptica* 2320; *well 6, no addition.*

reduced compared with the activity found when pertussigen is in combination with FHA or that pertussigen in combination with FHA is more effective. Nakase and Doi (22) found that purified FHA alone did not protect mice but that small amounts of pertussigen made these preparations protective. When the degree of contamination of FHA preparations was measured by the ability to sensitize mice to histamine, we found that our FHA preparations contained not more than 0.13% pertussigen (50% sensitizing dose of FHA was 2 μg/mouse; 50% sensitizing dose of pertussigen was 0.0027 μg/mouse). If this was the actual contamination, the results of the mouse protection test may indicate that either a very small amount of pertussigen (≃0.14 μg) combined with FHA is capable of stimulating immunity or that this small amount is capable of making FHA an effective immunogen. Since FHA is a very good antigen by itself, whereas pertussigen is a rather poor antigen, as judged by production of precipitins in rabbits, mice, and sheep (unpublished data), it seems likely that the adjuvant action of pertussigen was not responsible, but that pertussigen itself was the substance that became highly effective in combination with FHA. The other possibility is that pertussigen acted both as a specific immunogen and as a nonspecific factor by increasing the permeability of the blood-brain barrier to antibodies and immune cells with specificity to surface antigens of *B. pertussis* (17). If this is the case, surface antigens, such as FHA and agglutinogens, may be effective in preventing infection, provided that a small amount of pertussigen is also present. We have not yet shown this experimentally, but the results of Nakase and Doi (22) seemed to support this hypothesis. When detoxified by glutaraldehyde, pertussigen was protective and did not sensitize mice to histamine at the small doses tested, but we have not tested the histamine-sensitizing activities of the relatively large doses needed to protect mice.

The results obtained by passive protection tests also indicated the lack of protective activity of FHA and supported the contention that pertussigen is the mouse-protective antigen. Furthermore, the failure to immunize mice to *B. pertussis* with whole-cell vaccines of *B. bronchiseptica* and *B. parapertussis* (27), which produce FHA but not pertussigen, provided additional indirect support for the importance of pertussigen in the protection of mice against i.c. challenges with *B. pertussis*.

The data reported here do not necessarily mean that pertussigen is the protective antigen for humans. It may well be that various cell surface antigens are important in the induction of immunity in humans. Among these are FHA, the agglutinogens, and endotoxin. However, pertussigen should still be one of the prime suspects, because it protects mice, and this activity has been correlated with the protective potency of pertussis vaccines. Glutaraldehyde-treated pertussigen offers interesting possibilities since, at least in mice, it is nontoxic and has a much reduced histamine-sensitizing activity but retains its antigenicity. Detoxified pertussigen seems to be a candidate for the antigen protective against whooping cough.

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